Reproductive and Developmental Toxicology Course

Extended One Generation Reproductive Toxicity (EOGRTS) Testing: What is it? Pros and Cons, experience and future

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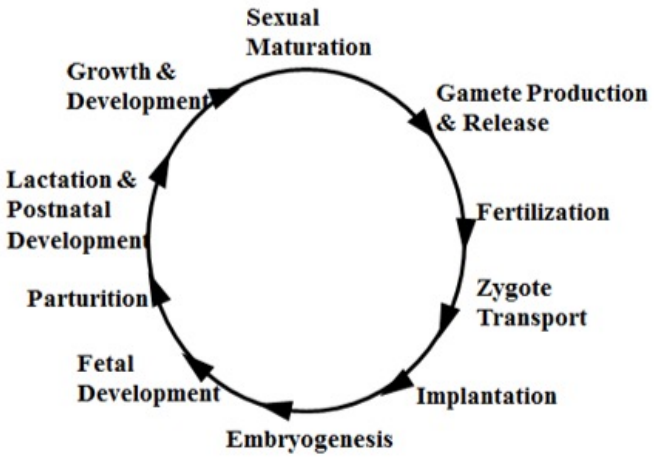
Outline

- Recap on OECD generational studies within the reproductive cycle
- History of the EOGRTS and when the study is required
- EOGRTS objectives, study design and endpoints, including different cohort descriptions
- Pros and cons of the EOGRTS
- Experience of the EOGRTS in Europe – the ECHA Review
- The future for the EOGRTS?

One or Multi-Generation tests – reminder

- Generation tests expose through the complete reproductive cycle



Pre-Mating to conception

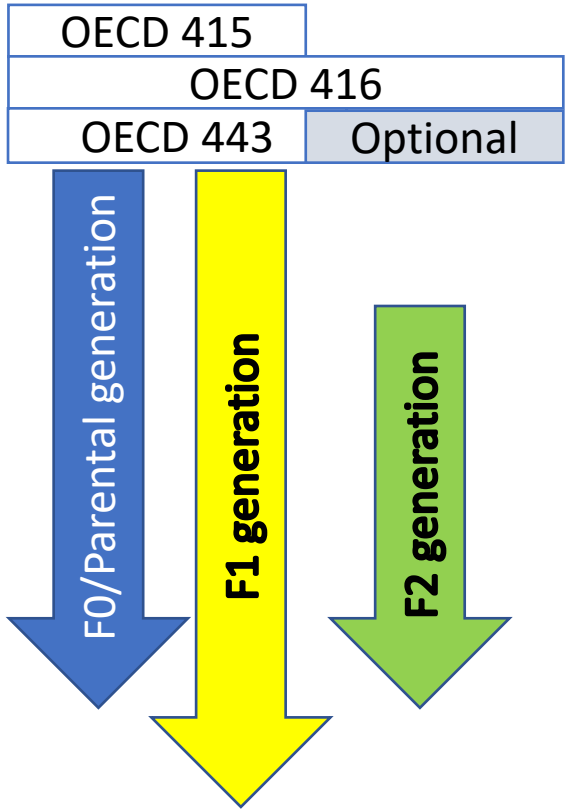
Conception to implantation

Implantation and organ formation

Organ formation to end of pregnancy

Birth to Weaning

Weaning to sexual maturity

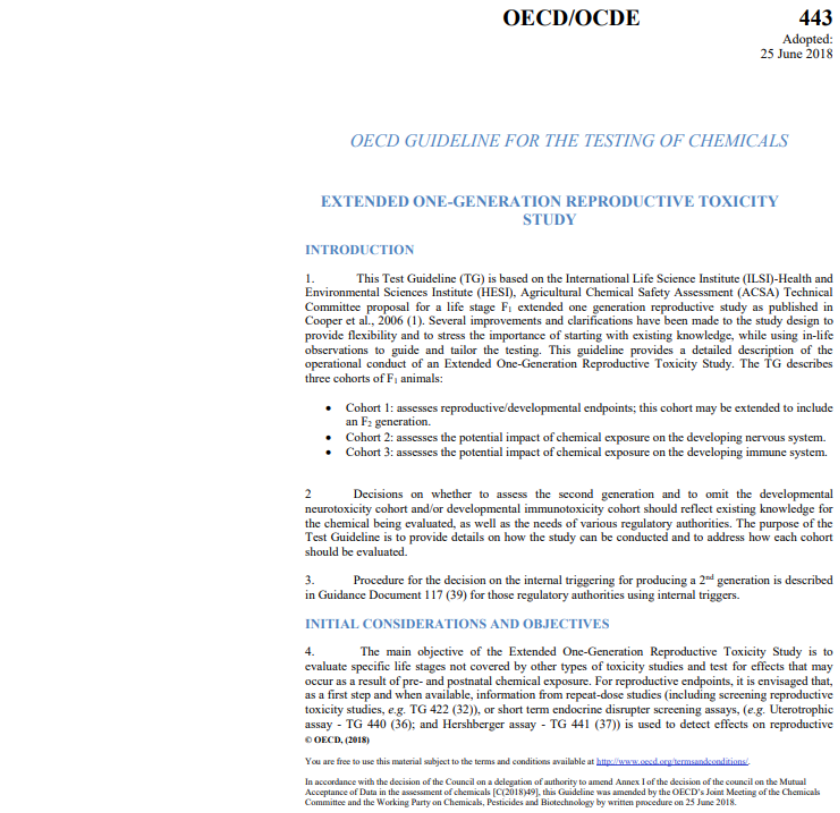


History of the OECD 443: Extended One Generation Reproductive Toxicity Study (EOGRTS)

EOGRTS adopted in 2011, based on a 2006 proposal from Agricultural Chemical Safety Assessment (ACSA) Technical Committee of the International Life Science Institute (ILSI)-Health and Environmental Sciences Institute (HESI)[1]

OECD 443 Extended One Generation Reproductive Toxicity study (EOGRTS) has largely replaced the OECD 416 to more comprehensively inform on reproductive toxicity. Now replaces OECD 416 in the standard information requirements for biocides and higher tonnage industrial chemicals. Expected for pesticide regulations to also be updated to replace the OECD 416 with the OECD 443

1 https://www.tandfonline.com/doi/full/10.1080/10408440500541367



OECD/OCDE **443**

Adopted:
25 June 2018

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

EXTENDED ONE-GENERATION REPRODUCTIVE TOXICITY STUDY

INTRODUCTION

1. This Test Guideline (TG) is based on the International Life Science Institute (ILSI)-Health and Environmental Sciences Institute (HESI), Agricultural Chemical Safety Assessment (ACSA) Technical Committee proposal for a life stage F_1 extended one generation reproductive study as published in Cooper et al., 2006 (1). Several improvements and clarifications have been made to the study design to provide flexibility and to stress the importance of starting with existing knowledge, while using in-life observations to guide and tailor the testing. This guideline provides a detailed description of the operational conduct of an Extended One-Generation Reproductive Toxicity Study. The TG describes three cohorts of F_1 animals:

- Cohort 1: assesses reproductive/developmental endpoints; this cohort may be extended to include an F_2 generation.
- Cohort 2: assesses the potential impact of chemical exposure on the developing nervous system.
- Cohort 3: assesses the potential impact of chemical exposure on the developing immune system.

2 Decisions on whether to assess the second generation and to omit the developmental neurotoxicity cohort and/or developmental immunotoxicity cohort should reflect existing knowledge for the chemical being evaluated, as well as the needs of various regulatory authorities. The purpose of the Test Guideline is to provide details on how the study can be conducted and to address how each cohort should be evaluated.

3. Procedure for the decision on the internal triggering for producing a 2nd generation is described in Guidance Document 117 (39) for those regulatory authorities using internal triggers.

INITIAL CONSIDERATIONS AND OBJECTIVES

4. The main objective of the Extended One-Generation Reproductive Toxicity Study is to evaluate specific life stages not covered by other types of toxicity studies and test for effects that may occur as a result of pre- and postnatal chemical exposure. For reproductive endpoints, it is envisaged that, as a first step and when available, information from repeat-dose studies (including screening reproductive toxicity studies, *e.g.* TG 422 (32)), or short term endocrine disrupter screening assays, (*e.g.* Uterotrophic assay - TG 440 (36); and Hershberger assay - TG 441 (37)) is used to detect effects on reproductive

In accordance with the decision of the Council on a delegation of authority to amend Annex I of the decision of the council on the Mutual Acceptance of Data in the assessment of chemicals [C(2018)49], this Guideline was amended by the OECD's Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology by written procedure on 25 June 2018.

https://www.oecd-ilibrary.org/environment/test-no-443-extended-one-generation-reproductive-toxicity-study_9789264185371-en

When is the EOGRTS (OECD 443) needed?

- A EOGRTS is a standard information requirement for biocidal active substances
- A EOGRTS is a standard information requirement for industrial chemicals manufactured or imported in quantities of ≥ 1000 tonnes or more
  - A EOGRTS can be a triggered study for industrial chemicals manufactured or imported ≤1000 tonnes if there are equivocal concerns for fertility identified
- A EOGRTS (or a 2-Generation Reproductive Toxicity Study - OECD 416) is a standard information requirement for a pesticide
- A EOGRTS is the most informative study on apical ED changes and can be conducted to investigate endocrine adversity if endocrine activity evident and the data set is only supported by an older generation study (predating v. 2001 of the OECD 416 study)

EOGRTS (OECD 443) study objectives

- *'to evaluate specific life stages not covered by other types of toxicity studies and test for effects that may occur as a result of pre- and postnatal chemical exposure. For reproductive endpoints, serves as a test for reproductive endpoints that require the interaction of males with females, females with conceptus, and females with offspring and the F1 generation until after sexual maturity'*
  - Evaluates the pre- and postnatal effects of chemicals on development as well as a thorough evaluation of systemic toxicity in pregnant and lactating females and young and adult offspring.
  - Detailed examination of key developmental endpoints, such as offspring viability, neonatal health, developmental status at birth, and physical and functional development until adulthood, is expected to identify specific target organs in the offspring.
  - In addition, the study will provide and/or confirm information about the effects of a test chemical on the integrity and performance of the adult male and female reproductive systems.
    - Specifically, but not exclusively, the following parameters are considered: gonadal function, the oestrous cycle, epididymal sperm maturation, mating behaviour, conception, pregnancy, parturition, and lactation.
  - Furthermore, the information obtained from the developmental neurotoxicity and developmental immunotoxicity assessments will characterize potential effects in those systems.

Study Design of the EOGRTS

- 2 or 10 week dosing period for Parental animals before mating. Administration contin result weani
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  - 2 – Developmental Neurotoxicity
  - 3 – Developmental Immunotoxicity
- Study informative on endocrine, development and Reproductive effects
  - Under REACH, OECD 443 primary purpose is to evaluate fertility and sexual behaviour, not development

Considered ‘Gold standard’ study for assessing reproductive toxicity and also the most informative study for evaluations of endocrine disrupting potential in Europe

generation	Cohort	Designation	Animals Cohort	maturation	t necropsy (weeks)
Target is 20 litters per group	1A	Reproductive	20 M + 20 F	Yes	13
	1B	Reproductive	20 M + 20 F	Yes	14 or 20-25 if triggered
	2A	Neurotoxicity	10 M + 10 F @	Yes	11-12
	2B	Neurotoxicity	10 M + 10 F @	No	3
	3	Immunotoxicity	10 M + 10 F @	Yes	8
	Surplus	Spares		No	3

@ one per litter and representative of 20 litters in total where possible

Endpoints of EOGRTS

Observation
Systemic toxicity (Clinical signs, body weight, food consumption, clinical pathology, macro- and micro-pathology)
Oestrous cycling and onset of first estrous after vaginal opening in both generations
Mating (fertility indices, time taken, duration of gestation)
Littering data (numbers of alive and dead pups, sex ratio, clinical condition, pup growth)
Anogenital distance
Nipple retention in males
Sexual maturity
Sperm evaluations
Thyroid hormone assessment
Splenic lymphocyte subpopulation analysis

Additional Cohorts – Cohort 1B : Mating for the 2nd Generation

- Cohort 1B (20 animals/sex/group) mated (between PND 90 to 120) if concern for reproductive hazard indicated by chemical class or previous data
- If additional mating, all procedures for F1/F2 generation must be identical
- Internal study triggers can also extend Cohort 1B to be mated (OECD Guidance document 117), e.g. equivocal data on sexual development, fertility indices, litter size etc
- Will not always have all data available before having to make decision on extension to mate, e.g. sperm data will be unknown
- ECHA/EFSA ED guidance document specifies EOGRTS must have mating to produce 2nd generation if study is to be considered informative for ED criteria – conflicting guidance
- Cost and time implications

Table 1. Criteria for assessing the second generation in the EOGRTS

Trigger Endpoints[a]	Recommendations
Adult Endpoints	
P Fertility (# implantations, pregnancy rate, gestational interval)	Mate F_1 in the absence of corresponding biologically relevant and dose-related changes in reproductive histopathology
F_1 Estrous Cycle Evaluation	Mate F_1 if biologically relevant and dose-related changes in estrous cycle length without severe toxicity in the dams[b]
Offspring Endpoints	
F_1 Litter parameters (litter size)	Mate F_1 if biologically relevant and dose-related decreases in litter size are seen in the absence of severe maternal toxicity or lethality[b]
F_1 Developmental landmarks (AGD, nipple retention, puberty onset, PPS, VO)	Mate F_1 if biologically relevant and dose-related effects in the absence of body weight-mediated changes in these parameters
↓ F_1 pup survival post-natally	Mate F_1 in the absence of severe maternal toxicity[b]
F_1 pup malformations	Mate F_1 in the absence of severe maternal toxicity[b]
↓ F_1 live birth index	Mate F_1 in the absence of severe maternal toxicity[b]
↓ F_1 pup body weight	Mate F_1, if pup body weight decrease is biologically relevant and in the absence of maternal body weight decrements

[a] Each endpoint will be available in sufficient time to determine whether or not the F1 should be mated.
[b] Type, incidence, magnitude and severity of effect(s) should be considered in relation to maternal toxicity.

Additional Cohorts – Cohort 2 : Developmental Neurotoxicity

- Cohort 2A (10 animals/sex/group):
  - Auditory startle (PND 24)
  - Functional observational battery (PND 63-75)
  - Motor activity (PND 63-75)
  - Neuropathology assessments (PND 75-90) – quantitative brain histomorphometry after perfusion fixation
- Cohort 2B (10 animals/sex/group – different litters to Cohort 2A):
  - Neuropathology assessments (PND 21-22)
- Growing requests from ECHA for learning and memory assessments in some circumstances, reflecting concern for hypothyroidism and impact on neurodevelopment
- Positive Historical Control data considered essential to interpret data and prove laboratory proficiency

Additional Cohorts – Cohort 3 : Developmental Immunotoxicity

- Cohort 3 (10 animals/sex/group – males and females from different litters to sample 20 litters in total)
- At PND 56 (±3 days), determination of primary IgM antibody response to a T-cell dependent antigen (TDAR)
  - Sheep Red Blood Cells (SRBC) or Keyhole Limpet Hemocyanin (KLH) are recommended antigens
  - Response determined by counting specific plaque-forming cells (PFC) in the spleen or by determining the titer of SRBC- or KLH-specific IgM antibody in the serum by ELISA, at the peak of the response (4-5 days after antigen immunization)
- Positive Control data considered essential to interpret data and prove laboratory proficiency

Additional guidance's

- Such a complex study has prompted a series of additional guidance documents
  - OECD Guidance Number 43 –Guidance document on Mammalian Reproductive Toxicity Testing and Assessment https://one.oecd.org/document/env/jm/mono(2008)16/en/pdf
  - OECD Guidance Number 117 –Guidance document on the Current Implementation of Internal Triggers in Test Guideline 443 for an Extended One Generation Reproductive Toxicity Study in the United States and Canada https://one.oecd.org/document/ENV/JM/MONO(2011)21/en/pdf
  - OECD Guidance Number 151 –Guidance document supporting OECD Test Guideline 443 on the Extended One Generation Reproductive Toxicity Test https://one.oecd.org/document/ENV/JM/MONO(2013)10/en/pdf

Pros of the EOGRTS

- It is argued the OECD 443 is beneficial over the OECD 416 due to:
  - More information being gained from less animals (up to 1200 less animals)
    - Greater statistical power (n=4 per litter retained)
    - More endocrine evaluations than OECD 416
      - Thyroid hormones and pathology
      - AGD and nipple retention as standard
      - More comprehensive oestrous cycle monitoring
      - Larger group size for sexual development assessment (n=60/group from 20 litters)
    - Focused investigations on immunotoxicity
    - Focused investigations on neurotoxicity (including brain histomorphometry and functional assessments)
  - Modular design means investigations are only included if needed and therefore fewer animals can be used if F2 investigations not necessary and less procedures will be conducted
  - More allowance for proof of lactational exposure to ensure exposure through all life stages

Cons of the EOGRTS

- Limited options to derive dose levels based on exposure arguments, can lead to unnecessarily excessive dose levels far in excess of likely human exposure
- Any effects on litter size or sex ratio are of greater consequence to the next generations selection
  - retaining at least 4M/4F/Litter instead of 1M/1F/Litter in the OECD 416
- Cohort 2 and 3 are too weakly powered (n=10) to be informative of anything but frank change
  - Naturally variable data e.g. brain histomorphometry
  - Little positive or historical control data to support evaluation
  - Would a OECD 426 DNT study be better?
- Thyroid hormone data too variable to be informative, even with n=20
  - Natural inter-animal variability exacerbated further by diurnal changes
  - Laboratory proficiency unclear in some cases (%CV >200%!)
- Inexperience with ‘new’ and variable endpoints, e.g. nipple retention, ano-genital distance
  - Regulatory hesitancy on how to interpret data

Cons of the EOGRTS cont.

- Widely considered the most complex of all *in vivo* studies within the data set
  - Logistically and scientifically challenging,
    - Increasing lab lead in times (+1 Year), Limited lab capacity
    - Huge cost of study (€750K to € 1.5 mil) in European and US labs. Numerous CROs offer study type but not all have relevant skills or experience
  - Complex reporting and data interpretation, increases reporting duration
- The second generations data were sometimes useful for classification purposes
  - Confirm previous generations findings to support 'true' effect instead of biological variability
  - Adversity only evident in F1 mating or second (F2) generation may be missed if no concerns in parental data. Rare but cannot be entirely discounted
- Mating to produce the second generation is 'triggered' but 2018 ECHA/EFSA ED identification guidance stipulates it should always be included to be informative for ED…conflicting guidance
- Escalating dose levels to comply with ECHA requirements will likely trigger more Cohort 1B extensions to mate due to secondary effects on sexual maturity delays

Experience with the EOGRT?

- In 2023, ECHA published a review of 55 EOGRTS[1] conducted for REACH to evaluate the performance of the EOGRTS, the design, conduct, analysis/reporting and if the results support hazard assessment in the EU regulatory context
  - Satellite-projects for:
    - Thyroid hormone measurements (France, Germany and ECHA)
    - Nipple retention and ano-genital distance (Denmark, France, Sweden and ECHA)
    - Follicular/corpora lutea count (France and ECHA)
    - Conclusions were expected for the end of 2023!
- Final report included:
    - 23/55 with extensions of Cohort 1B to produce F2 generations (8 were due to in-study triggers)
    - 24/55 with Cohort 2 (DNT)
    - 14/55 with Cohort 3 (DIT)

[1] https://echa.europa.eu/documents/10162/17228/final_report_eogrts_review_project_en.pdf/9d0b31f1-eff0-e9db-be8c-ac72d5e4b2e5?t=1679916891564

Main findings of the ECHA review

- The studies support the identification of SVHC
  - Around 30 % showed clear adverse effects on sexual function, fertility or development, influencing classification as reproduction toxicants
    - 14/55 – Repr. 2 (suspected to cause reproductive harm)
    - 18/55 – Repr. 1B (presumed to cause reproductive harm)
    - 16/55 – ED classification (T modality as most common but also EAS)
    - 14/55 – Supportive of STOT-RE
- The F2 generation can be effectively used to identify new effects and confirm findings
  - In 4/23, effects were only seen in the next generation
  - In 7/23, effects in the next generation lowered the NOAEL

Main findings of the ECHA review cont.

- In ECHAs opinion, 11/55 (20%) studies were underdosed to identify hazard and guide classification. Additional recommendations on dose setting now provided (https://www.flashpointsrl.com/app/uploads/2022/01/211221_echa_advice_dose__repro_en.pdf)
- Reiterates main purpose of the EOGRTS under REACH is to identify sexual function or fertility hazard, but it may also provide information on developmental toxicity or endocrine disruption.
  - Dose level selection focuses only on parental toxicity (to address fertility concern), irrespective of offspring toxicity. Change in focus risks the EOGRTS fulfilling all the objectives outlined in the OECD TG
  - ECETOC and HESI DART positions to be published soon
- Insufficiencies and methodological differences existed – Missing investigations, deviations and proficiency
  - More stringent proof of CRO proficiency now requested for a number of endpoints, e.g. positive control data for ED endpoints
  - CRO view of 'changing goal posts'

Comparison of systemic tox or reproductive tox NOAELs

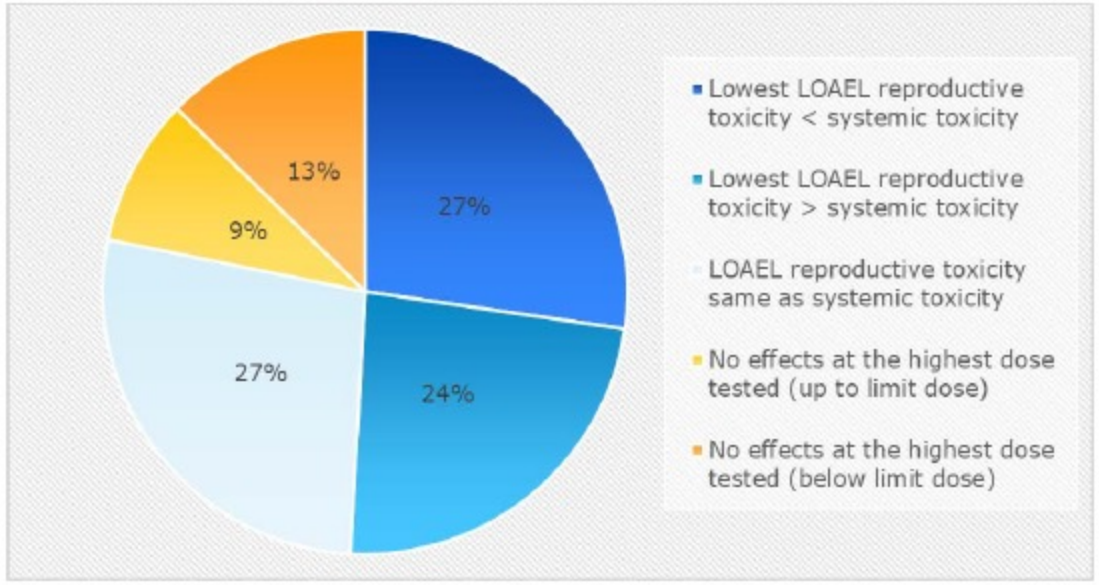

Figure 1: Percentage of cases (total of 55) where the lowest LOAEL for reproductive toxicity was higher, lower or the same as for the systemic toxicity or where no effects were reported at the highest dose tested.

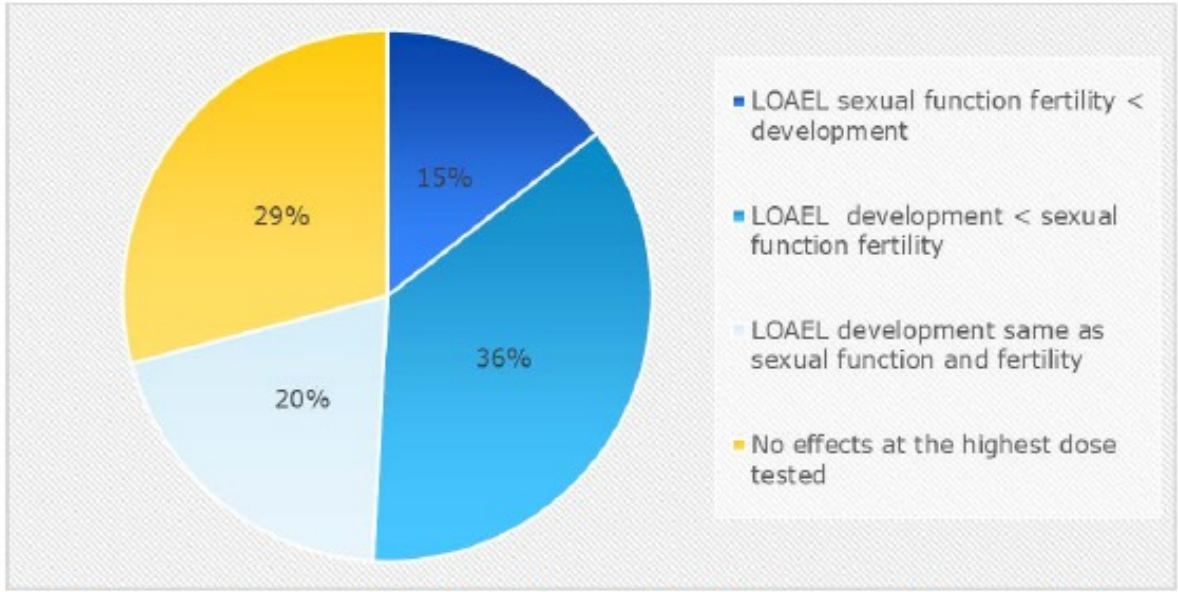

Figure 2: Percentage of cases (total of 55) where the lowest LOAEL for the effects on sexual function and fertility were higher, lower or the same as the lowest LOAEL for the effects on development or where no effects were reported at the highest dose on reproductive parameters.

- Roughly equal split between studies with more, less or same reproductive toxicity as systemic toxicity
- Developmental toxicity is more likely to drive reproductive NOAEL than sexual function or fertility when effects are seen (36% vs 15%)
  - Most common effect driving repro. NOAEL was < implantation sites
  - Most common effect driving dev. NOAEL was < implantation survival and post-natal mortality

Future for the EOGRTS?

- ECHA considers study design a success…its here to stay!
- Pesticidal active substance information requirements are likely to be updated to replace option of OECD 416 with OECD 443
- Dose level issues will be increasingly common
  - Potential rejection of existing studies – large consequences to repeating EOGRTS!
  - New studies adopting guidance to select high dose levels may result in insufficient offspring for next generation and excessive toxicity confounding data – risk for ED or Dev classification greater!
  - Internal study triggers more likely than before e.g. fertility issues such as oestrous cycle changes due to systemic toxicity or delayed sexual maturation due to offspring body weight changes
  - Already challenging and variable data set will only get more complex due to confounding toxicity…more expert toxicologist review
- Potential hesitancy to register substance in Europe due to risk to global registrations if EOGRTS is conducted to ECHA requirements – especially ED or developmental toxicity classification risk!
  - Risk for multiple generation studies? Lower dose levels for outside of Europe purposes?
- As CRO experience grows, historical control data will grow and potential regulatory uncertainties (or unfamiliarity with naturally variable data sets) will ease
- Cost of study only likely to increase as demand is greater than supply, but potentially more CROs will offer design
- Unknown consequences of the outcome from the satellite projects from the ECHA review

Summary

- EOGRTS are the largest and most complex of reproductive toxicity studies and they expose through the complete reproductive cycle, through at least one generation
- By using a modular design and utilising more animals per litter for different endpoints, the EOGRTS is considered to gain more information from fewer animals, benefiting science and ethics
- Numerous guidance documents exist to help navigate the complex design, which can also have in-study triggers which alter study design
- The EOGRTS is a complex study, with numerous challenges from both technical and regulatory aspects
- ECHA review has concluded that overall, the study is performing as intended and the inclusion of the next generation isn't always needed but can be informative where required
- The study presents many challenges which are only growing, including limited laboratory capacity and complex data interpretation at risk of confounding findings from excessive parental toxicity, highlighting importance of appropriate dose setting and expert interpretation

Questions

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